RESEARCH PAPER

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In Vitro Fermentation of caprine milk oligosaccharides by bifidobacteria isolated from breast-fed infants

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Abbreviations: ARDRA, Amplified rDNA restriction analysis; CMO, Caprine milk oligosaccharides; CMOF, Caprine milk oligosaccharide enriched fraction; FOS, Fructo-oligosaccharides; GIT, Gastrointestinal tract; GOS, Galacto-oligosaccharides; HMO, Human milk oligosaccharides; RAPD, Random amplified polymorphic DNA

This study was conducted to investigate the catabolism and fermentation of caprine milk oligosaccharides (CMO) by selected bifidobacteria isolated from 4 breast-fed infants. Seventeen bifidobacterial isolates consisting of 3 different species (*Bifidobacterium breve*, *Bifidobacterium longum* subsp. *longum* and *Bifidobacterium bifidum*) were investigated. A CMO-enriched fraction (CMOF) (50% oligosaccharides, 10% galacto-oligosaccharides (GOS), 20% lactose, 10% glucose and 10% galactose) from caprine cheese whey was added to a growth medium as a sole source of fermentable carbohydrate. The inclusion of the CMOF was associated with increased bifidobacterial growth for all strains compared to glucose, lactose, GOS, inulin, oligofructose, 3′-sialyl-lactose and 6′-sialyl-lactose. Only one *B. bifidum* strain (AGR2166) was able to utilize the sialyl-CMO, 3′-sialyl-lactose and 6′-sialyl-lactose, as carbohydrate sources. The inclusion of CMOF increased the production of acetic and lactic acid ($P < 0.001$) after 36 h of anaerobic fermentation at 37°C, when compared to other fermentable substrates. Two *B. bifidum* strains (AGR2166 and AGR2168) utilised CMO, contained in the CMOF, to a greater extent than *B. breve* or *B. longum* subsp *longum* isolates, and this increased CMO utilization was associated with enhanced sialidase activity. CMOF stimulated bifidobacterial growth when compared to other tested fermentable carbohydrates and also increased the consumption of mono- and disaccharides, such as galactose and lactose present in the CMOF. These findings indicate that the dietary consumption of CMO may stimulate the growth and metabolism of intestinal *Bifidobacteria* spp. including *B. bifidum* typically found in the large intestine of breast-fed infants.

Introduction

Evidence suggests that the microbial community of the human gastrointestinal tract (GIT) has a core function in maintaining host health by preventing the colonisation of pathogens,[1] degrading dietary compounds, producing metabolites able to be utilised by the host (e.g. short chain fatty acids, SCFA)[2] and maintaining mucosal immunity.[3] Particularly important in early life, the composition of the GIT microbiota influences the development and maturation of the foetal/neonatal GIT and consequently the overall health of the infant. During the perinatal period, the infant GIT is colonised by a relatively simple microbial community, initially derived from vaginal microbiota and maternal faeces.[4] The type of feeding (breast versus formula feeding), local environment, and antibiotic treatment may also play an important role in determining and maintaining the microbiota composition of the infant GIT.[5,6]

The GIT microbiota of breast-fed infants has long been thought to be dominated by bifidobacteria, compared to that of adults and formula-fed infants.[7] However, there are conflicting reports regarding differences in the relative abundance of these bacteria between breast- and formula-fed infants.[8-10] Moreover, although some studies have demonstrated a specific diversity of bifidobacterial species present in formula-fed infants is more like

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the adult bifidobacterial diversity,[11,12] culture or culture-independent methods, able to provide information on the identity and relative abundance of bifidobacteria to the species level, have not been used to compare the faecal microbiota of breast-fed vs. formula-fed infants. While the mechanisms for these differences in microbial colonisation and establishment are not fully understood, it is possible that the numbers of bifidobacteria species in breast-fed infants may be enhanced by the oligosaccharides in human milk (which collectively form the third largest solid component of milk (5 to 23 g l^{-1})[13] after fat and protein).

Milk oligosaccharides (sugar polymers, typically 3 to 10 units) have been studied extensively because of their marked influence on the GIT microbiota (i.e. prebiotic activity). Human milk contains a variety of oligosaccharides able to stimulate the growth of specific commensal GIT microbiota,[14] as well as stimulate the development of the immune system[15] and prevent adhesion of pathogens to epithelial tissues.[16] Infant-type bifidobacterial species such as *B. longum* subsp *longum*, *B. longum* subsp *infantis*, *B. bifidum* and *B. breve,* for example, contain enzymes specifically involved in the metabolism of human milk oligosaccharides (HMO).[17]

Modern infant formulas are increasingly supplemented with a mixture of plant derived oligosaccharides, such as fructo-oligosaccharides (FOS) and inulin (DP = 10 to 60), and lactose derived oligosaccharides, such as galacto-oligosaccharides (GOS).[18] These substrates elicit non-specific bifidobacterial growth (the "bifidogenic effect"),[19] and lack the complexity and diversity of HMO, so are unlikely to successfully mimic the structure specific effects of HMO. Fucosylated and sialylated HMO, for example, are complex oligosaccharides known to act as bacterial adhesin analogs and/or to mimic the receptors used by enteric pathogens to adhere to the surface of host epithelial cells.[16]

Sialyloligosaccharides support the growth of breast-fed, neonate specific commensal bacteria, such as *Bifidobacterium longum* subsp. *infantis* ATCC15697.[21] It has been suggested that these acidic oligosaccharides, in addition to eliciting a more specific bifidogenic effect, may combat influenza infections[22] and ulcers caused by *Helicobacter pylori.*[23] Other activities reported include regulation of inflammation by reducing adhesion of human leukocytes on activated endothelial cells, and promotion of commensal enteric bacterial proliferation.[24,25] The predominant forms of sialyloligosaccharides found in human milk, 3′- and 6′-galactosyl-lactose are also the most prevalent oligosaccharides in caprine colostrum, milk and whey. Therefore, the supplementation of infant formula with caprine milk oligosaccharides (CMO) is likely to stimulate the growth and metabolism of bifidobacterial strains typically found in breast-fed infants, and also to mimic the structure specific effects of HMO with associated infant health benefits. Understanding whether bifidobacterial strains from breast-fed infants are able to utilize CMO as a fermentable substrate is an important factor in the potential use of CMO as an infant formula supplement. To explore the effects of CMO on the growth and metabolism of specific postnatal bifidobacteria, this study aimed to investigate the ability of bifidobacterial strains, isolated from exclusively breast-fed neonates, to ferment a caprine milk oligosaccharide enriched fraction (CMOF) (especially sialyloligosaccharides), prepared from caprine cheese whey, using a recently published method,[26] and to produce SCFA (compared with other prebiotics or milk sugars).

Results

Genetic characterization of bifidobacterial strains

A total of 17 bifidobacterial strains were isolated from faecal samples of 4 exclusively breast-fed infants. The strains were positively identified by amplification and sequencing of a 498 bp region of the 16S rRNA gene corresponding to the V2 to V3 variable regions as *B. bifidum* (n = 4), *B. longum* (n = 6) and *B. breve* (n = 7) (**Fig. 1**). For the 6 *B. longum* strains, digestion of a partial 16S rRNA gene amplicon with Sau3AI was consistent with *B. longum* subsp *longum*.

RAPD sub-typing of the 17 bifidobacterial strains was undertaken to provide an indicative level of genetic heterogeneity that could be explored with further phenotypic assays. The dendrogram separated the 17 strains into 4 main clusters, broadly corresponding to the 3 different *Bifidobacteria* species (**Fig. 1**). A clear distinction between *B. longum* subsp *longum* strains isolated from infant 2 (AGR2170 to AGR2174) and infant 3 (AGR2176) was discernible by RAPD analysis.

Fermentation profile, SCFA and lactate analysis

All 17 strains were assessed for growth in media supplemented with glucose, CMOF (50% oligosaccharides, 10% GOS, 20% lactose, 10% glucose and 10% galactose),[26] combo, oligofructose, lactose, GOS, 3′-sialyl-lactose, 6’-sialyl-lactose, or no carbohydrate (**Fig. 2**). No significant growth ($OD_{600} < 0.5$) was observed from any bifidobacterial strains in the medium supplemented with inulin or with no added carbohydrate (data not shown).

In general, all strains reached higher optical densities at 16 h with CMOF as the sole carbohydrate source, compared to all the other carbohydrate sources ($P < 0.001$). The oligosaccharides 3’-sialyl-lactose and 6’-sialyl-lactose were fermented only by *B. bifidum* strain AGR2166 (**Fig. 2a**). *B. bifidum* strain AGR2166 fermented all tested carbohydrate substrates except oligofructose. The remaining *B. bifidum* strains (AGR2165, AGR2167 and AGR2168), were only able to ferment CMOF as a carbohydrate source (**Fig. 2b**). The *B. longum* subsp *longum* AGR2170, AGR2171, AGR2172, AGR2173, AGR2174 (**Fig. 2c**), AGR2176 (**Fig. 2d**) and *B. breve* AGR2169, AGR2177, AGR2178, AGR2179, AGR2181, AGR2183 (**Fig. 2e**), AGR2175 (**Fig. 2f**) strains reached an intermediate optical density ($OD_{600} = 1.5$) with oligofructose, and increased optical densities ($OD_{600} \geq 2$) with GOS, lactose, combo and glucose as the sole carbohydrate source, but with a different growth profile during the 48 h of fermentation.

A single strain representative of each fermentation profile was selected for quantifying SCFA production (**Fig. 3**, *B. bifidum* AGR2166 (a), AGR2168 (b); *B. longum* AGR2173 (c),

Cluster/group	Species	Strain number	Infant	Sialidase gene PCR amplification: Blon_2348 *B. infantis* ATCC 15697 (NC_011593.1)	BBPR_1794 *B. bifidum* PRL2010 (NC_014638.1)	BBPR_1793 *B. bifidum* PRL2010 (NC_014638.1)	BNR/Asp-box *B. breve* ACS-071-V-Sch8b (NC_017218.1)
A	*B. bifidum*	AGR2166*	1	-	+	+	-
		AGR2165	1	-	+	+	-
		AGR2167	1	-	+	+	-
		AGR2168*	1	-	+	+	-
B	*B. longum* subsp. *longum*	AGR2173*	2	-	-	-	-
		AGR2170	2	-	-	-	-
		AGR2171	2	-	-	-	-
		AGR2172	2	-	-	-	-
		AGR2174	2	-	-	-	-
C		AGR2176*	3	-	-	-	-
D	*B. breve*	AGR2179	4	-	-	-	+
		AGR2177*	3	-	-	-	+
		AGR2181	4	-	-	-	+
		AGR2178	3	-	-	-	+
		AGR2169	1	-	-	-	+
		AGR2183	4	-	-	-	+
		AGR2175*	3	-	-	-	+

Figure 1. Seventeen bifidobacterial strains isolated from 4 exclusively breast-fed infants were identified based on their 16S rRNA gene sequences. Dendrogram showing the relationships between the analyzed bacterial strains based on Dice similarity coefficients calculated from random amplification polymorphic DNA analysis data. The bar at the top of the diagram indicates the similarity index (maximum of 100%). PCR amplification and sequence analysis of sialidase genes was tested in all 17 bifidobacterial strains. (+) Identity with control sequence; (-) no amplification, or non-specific amplicon generated. * Strains chosen for further characterization by short chain fatty acid production and carbohydrate metabolism.

AGR2176 (d); *B. breve* AGR2177 (e), AGR2175 (f)). Only acetic and lactic acid were produced after 16 h and 36 h incubation for all the strains tested (**Fig. 3**). Acetate was shown to be present at time 0, due to the presence of sodium acetate (0.5%) in the basal media. The same media batch was used to investigate the growth of the different bacterial strains/carbohydrates for comparison purposes. All strains (except AGR2168), produced higher concentrations ($P <$ 0.001) of acetic and lactic acid in medium supplemented with CMOF at 36 h post-inoculation compared to the medium supplemented with the combo preparation (**Fig. 3**). AGR2168 only grew in the medium supplemented with CMOF, thus comparisons with the combo preparation were not possible. Formate was measured over the 36 h fermentation period but was produced at concentrations that were too low to quantify using our HP-LC methods.[27] Production of acetic and lactic acid by all the strains tested was associated with a decrease in overall pH of the culture over 36 h with the CMOF (**Fig. 4**). The drop in pH from 6.5 to 4.2 - 4.9 was correlated with the final OD of the culture (r= -0.95; $P = 0.001$), and the concentration of acetic (r= -0.85; P=0.02) and lactic acid (r= -0.83; $P = 0.03$) produced.

Bifidobacterial catabolism of oligosaccharides from CMOF

The initial relative abundance of several oligosaccharides associated with the CMOF, measured by LC-MS, was approximately 13% 3'- and/or 6'-galactosyl-lactose, 27% 3′- and/or 6′-sialyl-lactose, 32% 6'-Glycolyl-neuraminyl-lactose, 9% lacto-*N*-hexaose and less than 11% disialyl-*N*-lactose and 8% 6'-*N*-acetyL-glucosaminyl-lactose. All strains preferentially catabolised the 3'- and/or 6′-galactosyl-lactose and 3′- and/or 6′-sialyl-lactose (**Fig. 5**). *B. bifidum* AGR2166 had the highest levels of depletion (35 to 55%) of 3′- and/or 6′-galactosyl-lactose, 3′- and/or 6′-sialyl-lactose and 6′-glycolyl-neuraminyl-lactose. Disialyl-*N*-lactose and 6′-*N*-acetyL-glucosaminyl-lactose had the lowest depletion levels (<10%, similar among all strains). The depletion rate of all oligosaccharides, however, was different between strains from the same species (**Fig. 5**). *B. bifidum* AGR2166, for example, had the higher catabolism level among all strains, utilizing 33% of the total oligosaccharides identified in the CMOF, more pronounced then the reduction by *B. bifidum* AGR2168 (13%). A similar strain dependant variation of oligosaccharide catabolism was noted for *B. longum* subsp. *longum* AGR2173 (7%), AGR2176 (14%) and *B. breve* AGR2175 (5%) and AGR2177 (16%) strains. No correlation between the catabolism of CMO and bacterial growth were found after 36h of inoculation. This may have been due to the fermentation of other carbohydrates (e.g., mono- and di-saccharides) present in the CMOF that may have stimulated the bifidobacterial growth independent of CMO.

The concentrations of galactose, glucose, lactose, and GOS in a media supplemented with 1% CMOF were determined after 36 h of bifidobacterial incubation and compared to the concentrations immediately after inoculation (**Fig. 6**). These data indicate that lactose was depleted by all bifidobacterial

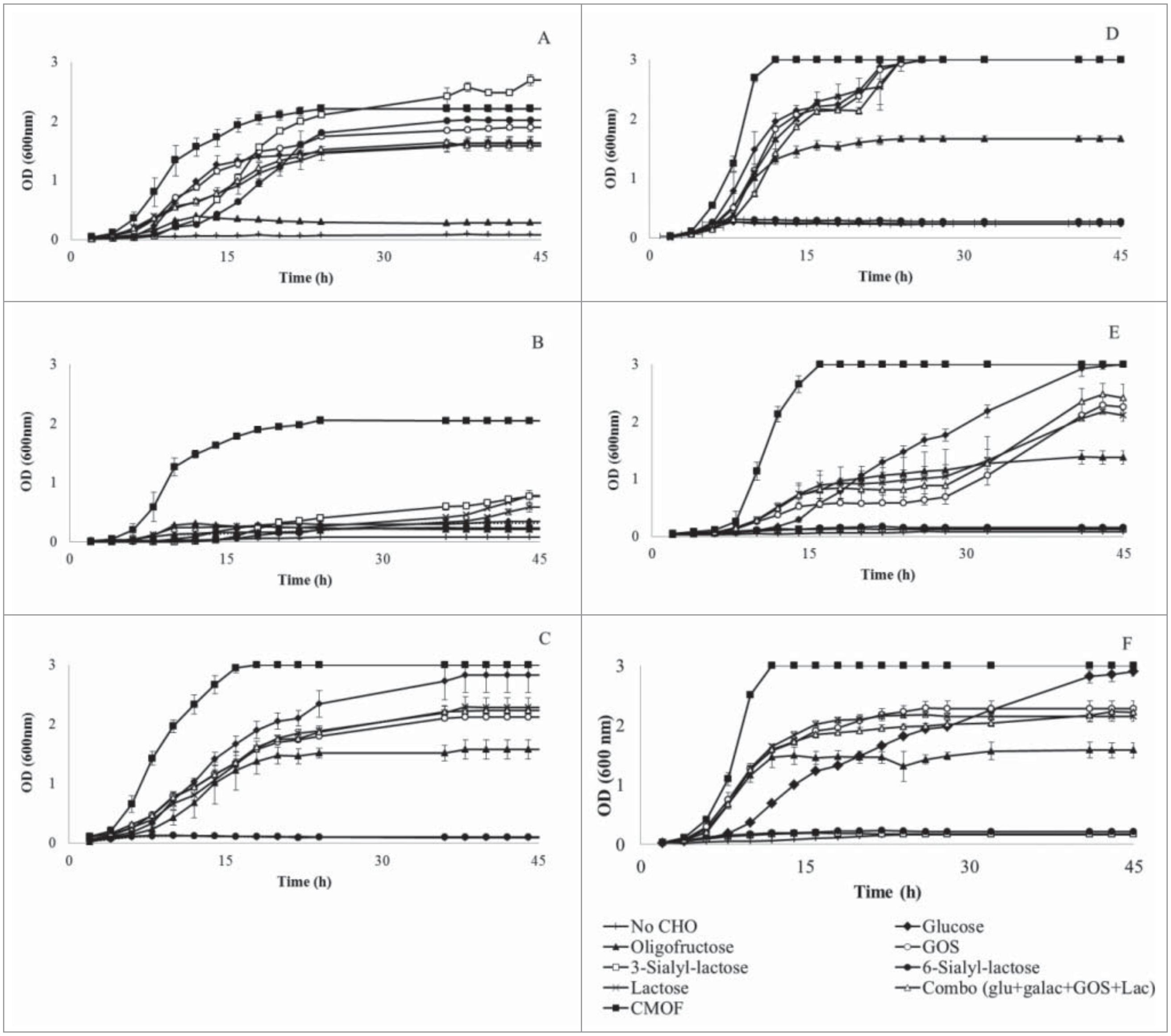

Figure 2. Growth profile of (A) *B. bifidum* AGR2166; (B) *B. bifidum* AGR2168 (similar to AGR2165 and AGR2167); (C) *B. longum* AGR2173 (similar to AGR2170, AGR2171, AGR2172 and AGR2174); (D) *B. longum* AGR2176; (E) *B. breve* AGR2177 (similar to AGR2169, AGR2178, AGR2179, AGR2181, AGR2183); (F) *B. breve* AGR2175. Each time point is an average of 3 replicates and the errors bars indicate standard deviation.

strains tested. The degradation of lactose, GOS, and CMOF were likely to have increased the overall concentrations of glucose and galactose in the media, and were not fully utilised by the end of the 36 h incubation. *B. bifidum* AGR2166 and AGR2168 had lower levels of glucose and higher levels of galactose in their media compared to pre-incubated media and to the other strains after CMOF fermentation. AGR2166 also had higher levels of lactose, compared to other strains, which may have been associated with oligosaccharide catabolism by this strain. The GOS concentration in the *B. longum* AGR2173 and *B. breve* AGR2175 media after incubation did not differ from uninoculated media; however, 50% of the GOS present in the pre-incubated media was fermented by the other strains.

Identification of genes encoding for exo-α-sialidase and associated sialidase activity

Sialidase activity of the bifidobacterial isolates on CMOF was assessed using molecular methods to demonstrate the presence of sialidase-encoding genes and by measuring sialidase activity using a fluorogenic substrate. The presence of 3 reported sialidase genes (BBPR_1793 and BBPR_1794 from *B. bifidum* PRL2010 and HMPREF92 28_0182 from *B. breve* ACS-071-V-Sch8b), were confirmed by PCR amplification and DNA sequencing in the *B. bifidum* and *B. breve* strains from this study (**Fig. 1**). Sialidase enzyme activity was also analyzed in 6 selected isolates (highlighted in **Fig. 1**); 2 *B. breve*, 2 *B. bifidum* and 2 *B. longum*. Only the *B. bifidum* isolates (AGR2166 and AGR2168) had cellular sialidase activity when grown in the presence of CMOF

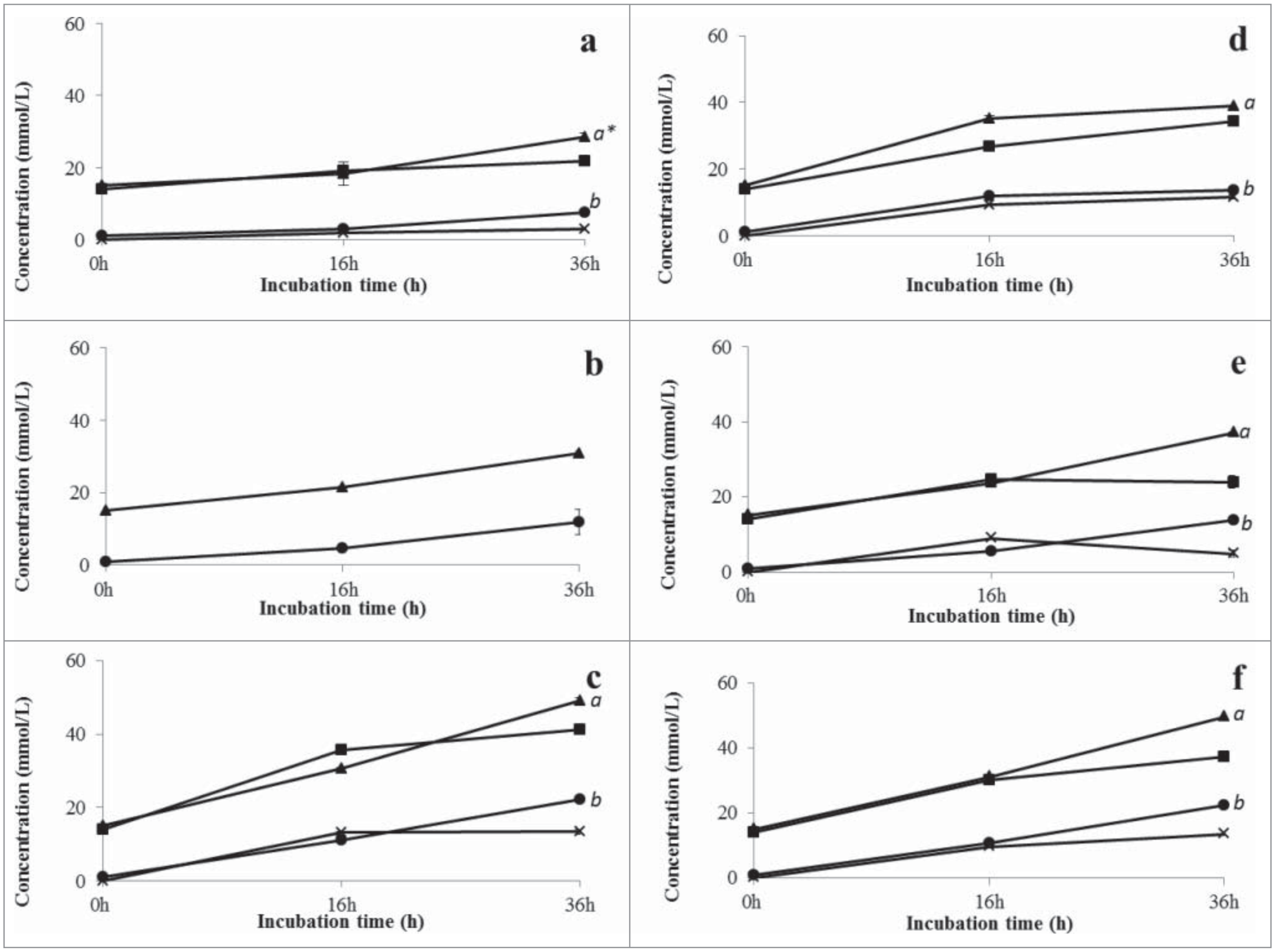

Figure 3. Acetic and lactic acid produced by bifidobacterial strains; (**a**) *B. bifidum* AGR2166; (**b**) AGR2168; (**c**) *B. longum* AGR2173; (**d**) AGR2176; (**e**) *B. breve* AGR2177; and (**f**) AGR2175. Each strain was grown in triplicate in semi-synthetic broth supplemented with 1% caprine milk oligosaccharides (determined from caprine milk oligosaccharide enriched fraction). Acetate and lactate were measured at 0 h, 16 h and 36 h of growth and compared to media supplemented with combo. ▲, acetate produced by caprine milk oligosaccharides enriched product fermentation; ■, acetate produced by combo fermentation; ●, lactate produced by caprine milk oligosaccharides enriched product fermentation; ×, lactate produced by combo fermentation. Each short chain fatty acids data point is an average of 3 replicates and the errors bars indicate standard deviation. * [a] Higher concentrations of acetate compared to combo ($P< 0.001$). [b] Higher concentrations of lactate compared to combo ($P < 0.001$).

(**Fig.** 7), although *B. bifidum* AGR2168 had only limited ability to ferment the CMOF sialyloligosaccharides, 3′- and 6′-sialyl-lactose as a sole source of carbon (**Fig. 2**). Sialidase activity from *B. bifidum* was mainly cell-associated, although approximately 12% residual activity was also detected in the culture supernatant of these 2 strains (**Fig. 7A**). *B. bifidum* cellular sialidase activity in AGR2166 and AGR2168 was induced by all 4 substrates examined, and was significantly enhanced ($P \leq 0.01$) in bacterial cell preparations taken from cultures grown in the presence of 3’- and 6′-sialyl-lactose when compared to CMOF and combo. Despite the presence of 3′ and 6′-sialyl-lactose in CMOF, there was no increase in sialidase activity of AGR2166 and AGR2168 grown in the presence of CMOF compared to the combo (**Fig. 7B**). Similar sialidase activity was observed from the culture supernatant taken from AGR2166 grown in the presence of 6’-sialyl-lactose and CMOF, and this sialidase activity was higher than combo and 3′-sialyl-lactose ($P < 0.001$). Despite the presence of a sialidase-encoding gene in the *B. breve* isolates examined, no sialidase activity was observed. However, in contrast to the sialidase proteins from *B. bifidum* that have signal peptide cleavage sites and transmembrane helices, no such structural characteristics were associated with the sialidase from *B. breve,* suggesting a potential intracellular localization. Intracellular sialidase activity was not determined.

Discussion

This study investigated the *in vitro* effects of a CMOF on the growth of selected bifidobacteria isolated from 4 exclusively breast-fed infants. The CMOF (containing high concentrations (46%) of sialyloligosaccharides), supported enhanced growth of

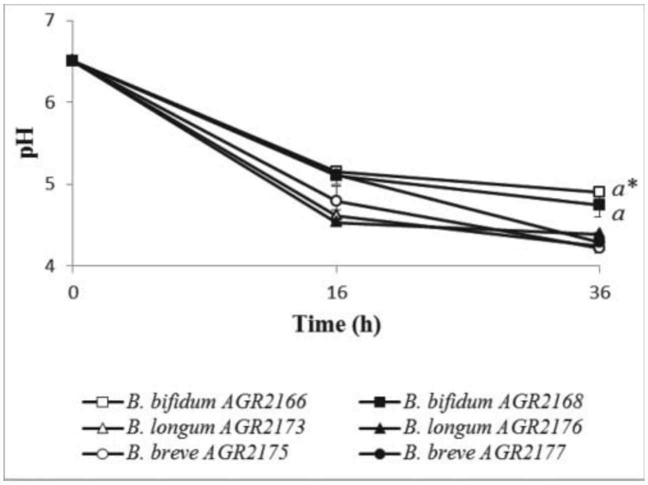

Figure 4. Change in pH associated with fermentation of caprine milk oligosaccharides enriched fraction by *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains. Each pH measurement is an average of 3 replicates and the errors bars indicate standard deviation. * ^{a}pH significantly higher ($P < 0.001$) at 36h compared to the other strains.

selected bifidobacteria strains isolated from breast-fed infants, and stimulated the *in vitro* production of lactate and SCFA, such as acetate. These results confirm the hypothesis, bifidobacteria isolated from the faeces of breast-fed infants are able to ferment CMOF, increasing bifidobacteria growth and metabolism.

In a recent study, CMO was shown to increase the growth of human faecal *Bifidobacterium* spp in anaerobic batch culture,[28] although the specificity of bifidobacterial CMO consumption was not investigated. The *B. breve*, *B. longum* and *B. bifidum* strains isolated in this work, together with *B. longum* subsp *infantis* and *B. adolescentis*, are among the most prevalent species found in infants independent of their feeding regime.[12,29] Although only a small number of bifidobacterial strains were selected from each infant, previous work suggests that the infant microbiota in the GIT is heterogeneous but is dominated by 3-5 different bifidobacterial species.[12,19] The RAPD analysis undertaken in this study broadly agrees with the well-recognized level of clonal heterogeneity demonstrated among the *B. breve*, *B. longum* and *B. bifidum* strains as determined by genetic fingerprinting methods such as ribosomal intergenic spacer analysis (RISA) and RAPD.[12,29]

Among the bifidobacterial species tested, *B. bifidum* (AGR2166) was shown to utilize both 3′- and 6′-sialyl-lactose as a sole carbon source to support growth (**Fig. 2**) with associated depletion of these same oligosaccharide isomers present in CMOF (**Fig. 5**). Enhanced depletion of 3′- and 6′-sialyl-lactose from CMOF by *B. bifidum* (AGR2166) was likely through cell-associated sialidase expression after induction with the same oligosaccharides (**Fig. 7**). *B. bifidum* (AGR2168), in contrast, displayed intermediate growth on 3′- sialyl-lactose (**Fig. 2b**) with partial (20%) utilization of 3′- and 6′-sialyl-lactose from CMOF (**Fig. 5**) with cell-associated sialidase expression (**Fig. 7**). These data largely agree with previous work[30] that suggests a surface or intracellular location for the sialidase enzyme on *B. bifidum.* The residual activity of sialidase found in the *B. bifidum* (AGR2166 and AGR2168) culture supernatant therefore may be associated with cell wall debris and/or released enzyme present within the culture supernatant.

B. longum and *B. breve* strains were unable to utilize 3′- and 6′-sialyl-lactose as a growth substrate when included as the only carbon source (**Fig. 2c to 2f**) and no expression of cell-associated sialidase was observed (**Fig. 7**). Limited depletion (5- 15%) of these oligosaccharides by *B. longum* and *B. breve* strains was detected (**Fig. 5**) however, when grown in a CMOF enriched media. This partial depletion may have occurred through incomplete catalysis of 3′- and 6′-sialyl-lactose without fermentation of any resulting breakdown products or that these sugars were selectively adsorbed to the bacterial cells that were present in the media, which were then removed prior to analysis.[31] Contrasting oligosaccharide depletion observed between strains of the same species was in accordance with inter-strain heterogeneity shown in the RAPD analyses. *B. breve* AGR2175 and AGR2177, for example, both isolated from the same infant (3) and with similar growth profiles (**Fig. 2**), showed different oligosaccharides catabolism profiles (**Fig. 5**) which might indicate differential regulation or expression of enzymes involved in carbohydrate metabolism.

Augmented microbial biomass associated with enhanced growth and fermentation of CMOF increased microbial fermentation end products such as acetate and lactate. These data agree with a previous study, where CMO was shown to increase the production of acetate, lactate and propionate in anaerobic batch culture inoculated with human faeces.[28] Formate may also be produced as an end-product of bifidobacterial fermentation with the inclusion of fructose or OF as the main carbohydrate source[27] but was produced at levels that were too low to detect using HP-LC.

An absolute measurement of CMO utilization in this study was impossible due to the high concentrations (50%) of lactose, GOS, glucose and galactose in the CMOF. However, when used as a sole carbohydrate source, lactose, GOS, glucose and galactose did not support enhanced bacterial growth when compared to the CMOF. It is likely that the CMO component of the overall CMOF, is not only a fermentable substrate, but also stimulates the utilization of other simpler carbohydrates. The GlcNAc-containing oligosaccharides (6′-*N*-acetyL-glucosaminyl-lactose and lacto-*N*-hexaose), for example, have been reported as a growth factor stimulating lactose utilization by *B. bifidum.*[38] The mechanism through which these oligosaccharides are used remains to be identified, but studies on the utilization of HMO may provide some clues.[14,39] Certain bifidobacterial strains such as *B. bifidum* NCIMB41171 have the ability to synthesize long chain carbohydrates (such as GOS) from lactose and galactose using the transglycosylic activity of β-galactosidase.[40] Thus, it is difficult to precisely determine how much lactose was degraded to glucose and galactose through the hydrolytic activity of β-galactosidase, and how much GOS, if any, was produced by transglycosylic activity of β-galactosidase.[41,42]

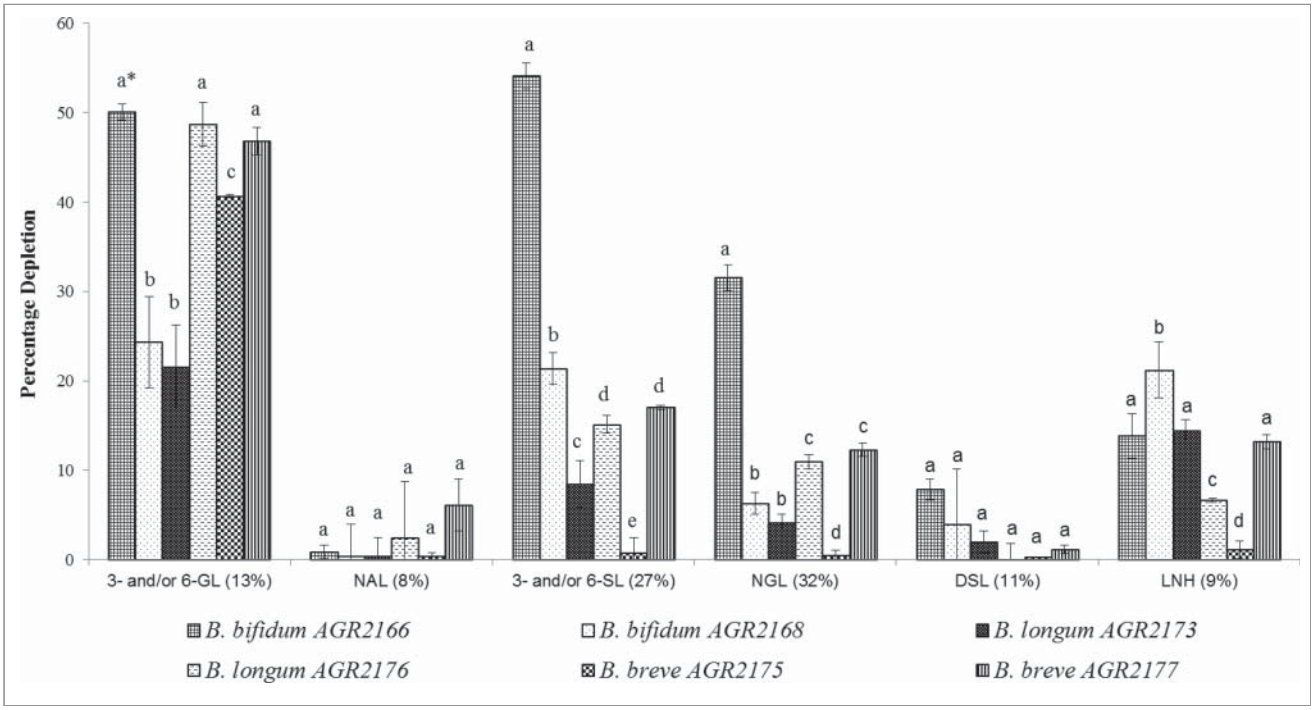

Figure 5. Percentage of oligosaccharide depletion by *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains after 36 h of growth in semi-synthetic broth supplemented with 1% of caprine milk oligosaccharide (determined from caprine milk oligosaccharide enriched fraction), when compared to the uninoculated control media. The oligosaccharides are represented by their abbreviation and their relative initial abundance is shown in brackets. 3'- and/or 6'-galactosyl-lactose (3-GL and/or 6-GL), 3'- and/or 6'-sialyl-lactose (3-SL and/or 6-SL), 6'-glycolyl-neuraminyl-lactose (NGL), lacto-*N*-hexaose (LNH), disialyl-*N*-lactose (DSL), 6'-*N*-acetyL-glucosaminyl-lactose (NAL). Each strain was incubated in triplicate and errors bars show the standard deviation of depletion. * [a, b, c] Bars with dissimilar letters differ significantly in depletion within each oligosaccharide ($P < 0.001$).

Lactose, the core of all HMO and CMO, and the main structure of galactosyl-lactose, is likely to be degraded to galactose and glucose in a catabolic reaction that requires β-galactosidase activity. *B. bifidum*, for example, contains both extracellular and intracellular β-galactosidases.[43] *B. breve*[41] and *B. longum* subsp *longum*,[43] on the other hand, have been reported to contain only intracellular β-galactosidases, and the high utilization of lactose by these strains may indicate that lactose is likely to be actively transported into the cells by a yet unidentified transporter. Although GOS is also hydrolysed to glucose and galactose by β-galactosidase, different strains have been shown to have differential consumption of selected GOS with different DPs.[44] The infant isolates (*B. longum* subsp *infantis* and *B. breve*) are able to more efficiently consume the GOS species with DP from 3 to 8, while *B. adolescentis* and *B. longum* subsp. *longum* exhibited differential consumption of selected DP.[44] These contrasting HMO and CMO utilisations suggest that niche specific adaptation abilities exist among various bifidobacterial species and strains, and with other components of the microbiota of the GIT. These complex effects cannot be reproduced by simple carbohydrate structures most often used as prebiotics.

The ability of the bifidobacterial strains to utilize the different carbohydrates present in CMOF and/or stimulate the consumption of other carbohydrate sources is important to determine the effects of these milk components in the GIT microbiota. Although more than 8% of the identified genes from bifidobacterial genomes are predicted to be involved in carbohydrate metabolism, the ability to metabolise certain complex milk oligosaccharides is species and strain specific.[45,46] Analysis of the genes involved in carbohydrate utilization indicate that *B. bifidum* (JCM1254 and JCM7004) contain genes that encode specialized enzymes associated with the extracellular deglycosylation of milk oligosaccharides, including extracellular α-fucosidases,[47] β-galactosidases, β-N-acetylglucosaminidases[48] and α-sialidases,[30] which efficiently remove monosaccharides from complex milk oligosaccharides. *B. bifidum* and *B. longum*[49] also contain a membrane enzyme, lacto-N-biosidase, responsible for the cleavage of the bifidogenic HMO lacto-n-tetraose to lacto-n-biose[50] and lactose. The mono- and disaccharides released by this endoglycosidase (especially lacto-n-biose), are internalised by family 1 solute binding proteins, and metabolised. Family 1 solute binding proteins are part of a gene cluster conserved across all infant GIT-associated bifidobacteria, including *B. bifidum*, *B. infantis*, *B. longum* and *B. breve* isolates.[39,51] The same enzyme degradation mechanism may be responsible for the utilization of lacto-N-hexaose present in CMO.

None of the selected bifidobacterial strains were able to utilize inulin as the sole carbohydrate source, but all except the *B. bifidum* strains were able to utilize oligofructose. The DP (oligofructose DP 2-10; HP inulin DP 11-60) is likely to influence the ability of

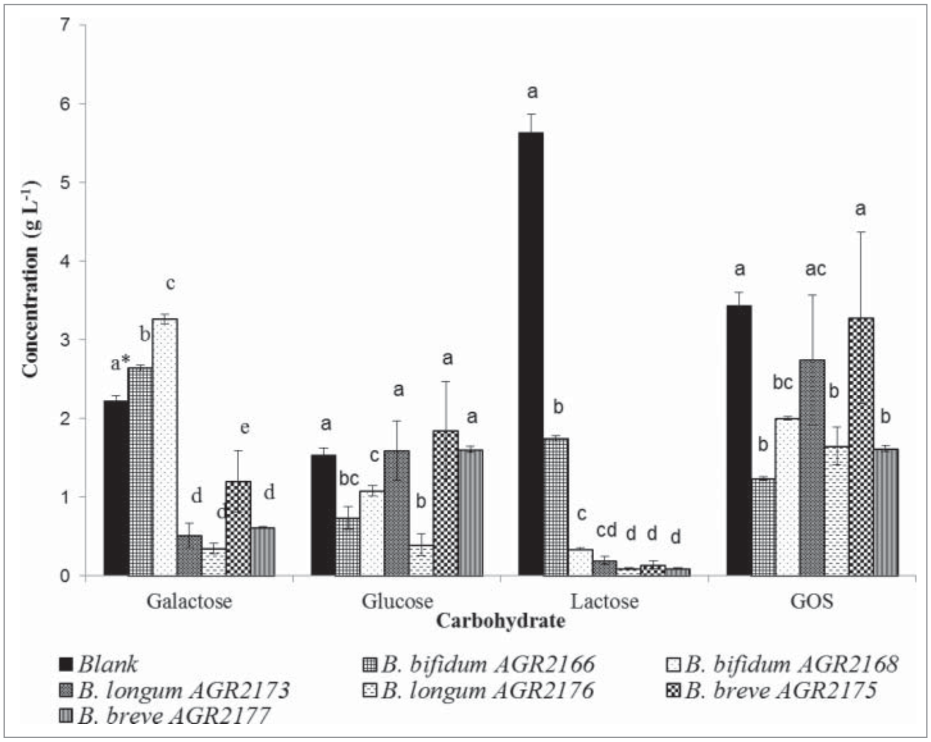

Figure 6. Total residual carbohydrate remaining in semi-synthetic media supplemented with 1% of caprine milk oligosaccharides (determined from caprine milk oligosaccharide enriched fraction) after 36 h of fermentation by *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains. Each strain was incubated in triplicate and errors bars show the standard deviation of remaining carbohydrate. * [a, b, c] Bars with dissimilar letters differ significantly ($P < 0.001$), within each carbohydrate.

bifidobacterial strains to utilize FOS as the sole carbon source.[52] However, bifidobacteria present in breast-fed infants may also be selectively stimulated by milk oligosaccharides instead of plant derived oligosaccharides. Previous studies confirmed the poor growth of *B. bifidum* strains on inulin type fructans,[53,54] but strain differences in β-fructofuranosidase production levels have been reported.[55] After weaning, with the introduction of plant derived foods, *B. bifidum* strains are likely to benefit indirectly from the fermentation of inulin type fructans by other members of the GIT microbiota through the lowering of the GIT pH, or the increased availability of monosaccharides as substrates.[53]

In conclusion, faecal bifidobacteria species isolated from breast-fed infants are heterogeneous at the genetic level based on RAPD profiles, but also at the level of substrate utilization through the differing depletion of certain carbohydrate components of the CMO preparation including some oligosaccharides. CMOF was able to stimulate the growth of bifidobacteria commonly found in the GIT of breast-fed infants. CMO contained in the CMOF may also have stimulated the consumption of lactose, glucose, galactose and GOS. Comparing the selected strains, *B. bifidum* were better able to ferment CMOF, especially the sialyloligosaccharides, which may indicate that *in vivo*, this strain may benefit from CMO consumption. Defining and linking the utilization of specific oligosaccharide structures, such as the sialyloligosaccharides, to cultured bacteria will provide a scientific path for targeting infant health by establishing protective microbial communities, beneficial to their hosts and potentially applicable to different stages of human life and health states.

Material and Methods

Isolation of bifidobacteria

Collection of faecal samples from healthy breast-fed infants was approved by the local human ethics committee (Massey University, Palmerston North, New Zealand). Bifidobacteria were isolated from faeces obtained from freshly soiled diaper/nappy of 4 exclusively breast-fed neonates on modified TPY agar (MTPY).[56] The plates were incubated anaerobically (93% CO_2, 7% H_2) at 37°C for 48 h. Bacterial colonies were then individually picked, subcultured on fresh MTPY agar plates to obtain single colonies and again on anaerobic De Man-Rogosa-Sharpe (MRS, Oxoid, CM0361) agar slopes (pH 6.5 – 7.0) supplemented with L-cysteine-hydrochloride (0.5 g l^{-1}), at 37°C for 48 h, for storage at −80°C until further analysis.

Bifidobacterial characterization

The identity of the bifidobacterial isolates was confirmed using PCR and 16S rRNA gene sequencing using bif 164 (5-GGG TGG TAA TGC CGG ATG-3) and bif 662 (5- CCA CCG TTA CAC CGG GAA -3) primers.[57] Sequencing of the PCR product (15 ng) was performed using the bif 164 or bif 662 primers (3.2 nM) and 16S rRNA gene sequences were compared with known bacterial sequences available from GenBank database using BLAST.

Randomly amplified polymorphic DNA

Random amplified polymorphic DNA (RAPD) PCR was performed using 7 random decamer primers: P2 5′-GAT CGG ACG G-3′, P15 (5′ CTG GGC ACG A 3′), P16 (5′ TCG CCA GCC A 3′), P17 (5′CAG ACA AGC C 3′),[58] PER1 (5′AAG AGC CCG T 3′),[59] and CC1 (5′AGC AGC GTG G 3′),[60] CORR1 5′-TGC TCT GCC C-3′.[61] The amplifications were carried out using 100 ng template DNA and a dendogram was generated from the RAPD profiles generated with the 7 random primers using Bionumerics 4.0 (Applied Maths). Profiles were compared using Dice's Similarity Coefficient at a tolerance of 2%.

control DNA was kindly gifted by Professor Gerald Tannock (University of Otago, New Zealand).

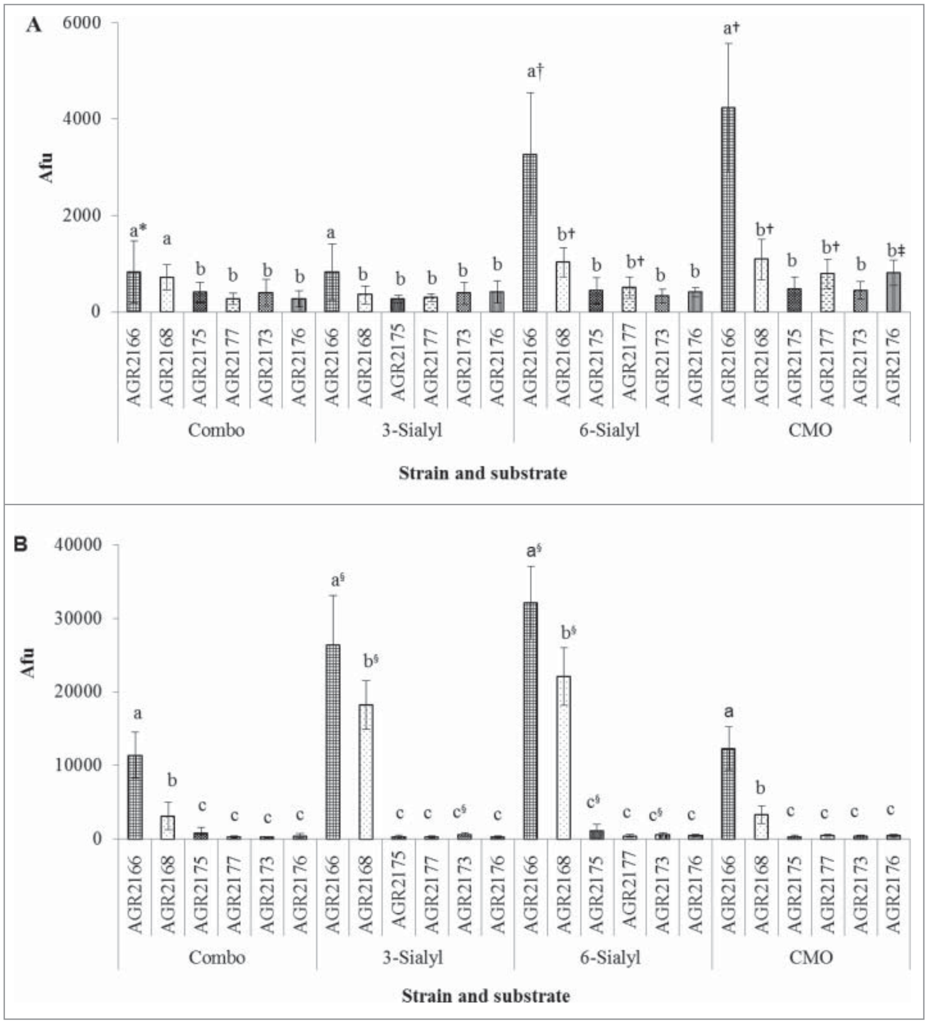

Figure 7. Sialidase activity associated with *B. bifidum* (AGR2166 and AGR2168), *B. longum* (AGR2173 and AGR2176) and *B. breve* (AGR2175 and AGR2177) strains after growth in semi-synthetic media supplemented with 1% (w/v) final concentration of combo, 3′-sialyl-lactose, 6′-sialyl-lactose and caprine milk oligosaccharides (determined from caprine milk oligosaccharide enriched fraction). Sialidase activity associated with culture supernatant and cells alone was measured by absolute fluorescence units (Afu) produced by 4-methylumbelliferone released during MUN hydrolyses. (**A**) Culture fluid sialidase activity; (**B**) Cellular sialidase activity. Six separate cultures of each of 2 *B. bifidum*, 2 *B. longum*, and 2 *B. breve* strains were examined, with each biological replicate assessed in triplicate. * [a, b, c] Bars with dissimilar letters differ significantly ($P < 0.001$), within each substrate. †Caprine milk oligosaccharides enriched product and 6′-sialyl-lactose with increased sialidase activity compared to combo and 3′-sialyl-lactose ($P \leq 0.001$). ‡Caprine milk oligosaccharides enriched product with increased sialidase activity compared to the other substrates ($P < 0.001$). §Substrate with increased sialidase activity ($P \leq 0.01$) compared to caprine milk oligosaccharides enriched product and combo.

Carbohydrate fermentation and measurement of SCFA and lactate

To investigate the growth profiles of bifidobacterial strains, each strain was inoculated into MRS broth supplemented with 0.05% (w/v) L-cysteine and incubated at 37°C for 36 h until late logarithmic/early stationary phase was reached. Each strain was then subcultured (100 μl) into a defined semi-synthetic medium[63] supplemented with a carbohydrate source at a final concentration of 1% (w/v). The carbohydrates added were either CMOF (5 g l^{-1} caprine milk oligosaccharides, 1 g l^{-1} GOS, 2 g l^{-1} lactose, 1 g l^{-1} glucose and 1 g l^{-1} galactose) prepared from caprine cheese whey, and characterized as described previously[26]; combo, identical to CMOF but lacking CMO, (1 g l^{-1} GOS, 2 g l^{-1} lactose, 1 g l^{-1} glucose and 1 g l^{-1} galactose); 10 g l^{-1} of glucose (BDH), lactose (BDH), oligofructose P95 (BENEO-Orafti; average degree of polymerisation [DP] of 4), inulin HP (BENEO-Orafti; average DP of 25), galacto-oligosaccharide (GOS) (TOS-100, Yakult; DP <8), 3′-syalyl-lactose (Carbosynth, OS31041) or 6′-syalyl-lactose (Carbosynth, OS04398). The dominant oligosaccharides present in the CMOF were 3′- and/or 6’-galactosyl-lactose (12%, DP = 3), 3′- and/or 6′-sialyl-lactose (27%, DP= 3), 6′-glycolyl-neuraminyl-lactose (32%, DP= 3), lacto-*N*-hexaose (9%, DP= 6), disialyl-*N*-lactose (11%, DP= 4), 6′-*N*-acetyL-glucosaminyl-lactose (7%, DP = 3). Each bifidobacterial strain was grown in triplicate in 5 mL of semi-synthetic broth, and cultured anaerobically for 45 h at 37°C. Cell growth was measured 2 hourly by taking optical density measurements at a wavelength of 600 nm on a spectrophotometer (Ultrospec 1100 pro, Amersham Biosciences).

Fermentation reactions from 6 strains (2 *B. bifidum*, 2 *B. longum*, and 2 *B. breve*), selected due to their optimum growth in CMO-supplemented medium, were evaluated for SCFA production. Anaerobic growth of each strain in 6 different broth cultures supplemented with CMO (10 g l^{-1}, 1% [w/v] final concentration contained in CMOF), and combo (10 g l^{-1}, 1% [w/v]), was

Amplified rDNA restriction analysis (ARDRA) of *B. longum* subsp *longum* versus *infantis*

PCR amplification of a 914 bp DNA fragment and restriction endonuclease digestion with Sau3AI (New England Biolabs, R0169S) was used to differentiate *B. longum* subsp *longum* from *B. longum* subsp *infantis* as described previously.[62] The *B. longum* subsp *infantis* strain JCM 10088

Table 1. Sialidase genes and primers used to screen for sialidase domains in the bifidobacterial isolates. (**A**) Sialidase protein characteristics, (**B**) Predicted cellular localization, based on the presence of export signal, transmembrane domain or cell wall anchor motifs and primers.

A)

Origin	GenBank accession number	Gene Tag	Length (aa)	GH33 region (aa)	Signal peptide cleavage site*	Transmembrane helices†
B. longum subsp. *infantis* ATCC15697	ACJ53406	Blon_2348	394	35-368	None	None
B. breve ACS-071-V-Sch8b	AEF27628	HMPREF9228_0182	763	325-730	None	None
B. bifidum PRL2010	ADP36806	BBPR_1793	1795	325-650	Between aa 39-40	Two between aa 17-39 and aa 1767-1789
B. bifidum PRL2010	ADP36807	BBPR_1794	834	190-510	Between aa 35-36	Two between aa 13-35 and aa 806-828

B)

Origin	Predicted localization	Primer name	Sequence	Amplicon length	Region amplified
B. longum subsp *infantis* ATCC15697	Intracellular	Sialil-Inf	F. 5′-TACTGTGTGCGGCGCGAACC R. 5′-CAGACAGCGGAAAACCGCCGA	1136bp	33-1168bp
B. breve ACS-071-V-Sch8b	Intracellular	Sialil-Br	F. 5′-GCGGTGCGGTGGACATCTAT R. 5′-CAGCCGACTTCACTCCGAA	1527bp	737-2263bp
B. bifidum PRL2010	Extracellular	Sialil-Bif5	F. 5′-GCGACCACTCAGGACGGCAC R. 5′-TCCGAGATCGCAACGCGACG	1198bp	448-1645bp
B. bifidum PRL2010	Extracellular	Sialil-Bif2	F. 5′-GCTGCATGCGGTCGTCGTCA R. 5′-TCGTGGCGTTGGCATTCGCA	1049bp	915-1963bp

*[66]

†TMHMM Server v. 2.0, http://www.cbs.dtu.dk/services/TMHMM/

assessed in triplicate at 37°C. At 16 h (mid logarithmic phase) and 36 h (stationary phase) after inoculation, broths were centrifuged for 10 min at 3000 x g at 4°C, and the pH were measured using a PHM62 pH meter (Radiometer Pacific). Aliquots of the fermentation liquid were filtered using 0.22 μm filters (Millipore, SLGVV255F) and the concentration of formic acid, acetic acid, propionic acid, lactic acid, isobutyric acid (2-methylpropanoic acid), butyric acid, isovaleric acid (3-methylbutanoic acid), valeric acid (pentanoic acid), and caproic acid (hexanoic acid) were determined using HPLC.

Utilization of CMOF by bifidobacteria

The utilization of CMO (10 g l^{-1}, final concentration 1% [w/v]), GOS (2 g l^{-1}), lactose (4 g l^{-1}), glucose (2 g l^{-1}) and galactose (2 g l^{-1}) present in the CMOF was assessed in 6 bifidobacterial strains (2 *B. bifidum*, 2 *B. longum*, and 2 *B. breve* strains) selected on the basis of contrasting growth profiles. Each strain was incubated in triplicate, and culture supernatants (50 μl of each tube) taken immediately after inoculation, and at stationary phase (36 h), were analyzed by LC-MS and HPLC to evaluate carbohydrate depletion. The depletion of specific oligosaccharides present in CMOF was analyzed by the intensity of their specific masses using LC-MS data and reported as percentage of depletion compared to the concentrations in the control. The HPLC and LC-MS methods used were those described previously.[26]

Bifidobacterial exo-α-sialidase genes

The presence of sialidase genes described for *B. longum* and *B. bifidum*[46,64] and a putative *B. breve* (NC_017218.1) sialidase gene were evaluated in the 17 bifidobacterial strains. PCR primers were designed to amplify the DNA sequence encoding the glycosyl hydrolase (GH) family 33 domain of the sialidase-encoding genes (**Table 1**) from *B. longum*, *B. bifidum* and *B. breve*.

Sialidase assay

The sialidase activity of selected bifidobacteria was determined using the fluorescent substrate 4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid (4MU-Neu5Ac) (Sigma, M8639) as described previously.[30] Briefly, bifidobacterial strains were grown anaerobically in 5 ml of a semi-synthetic medium[63] supplemented with 10 g l^{-1} CMO (contained in CMOF), combo, or of 3’-syalyl-lactose or 6’-syalyl-lactose as the sole carbohydrate source for 24 h at 37°C. Six separate cultures of each of 2 *B. bifidum*, 2 *B. longum*, and 2 *B. breve* strains were examined, with each biological replicate assessed in triplicate. Enzyme activities from washed bacterial cells and culture supernatant were determined by comparing absolute fluorescence units (Afu) minus the blank, as described previously.[65]

Statistical analysis

Bacterial growth, SCFA profiles and sialidase production at 36 h of fermentation for each substrate were tested for normality and homogeneity of variances and compared by one-way analysis of variance (ANOVA) using GenStat (15th edition SPS). Differences were considered significant at $P \leq 0.05$. To identify the correlation between bacterial growth, pH and SCFA production, Pearson’s Rank correlation factors and P values were calculated using GenStat (15th edition SPS). Correlations were considered significant if $P \leq 0.05$.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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References

1. Kamada N, Chen GY, Inohara N, Nunez G. Control of pathogens and pathobionts by the gut microbiota. Nat Immunol 2013; 14:685-90; PMID:23778796; http://dx.doi.org/10.1038/ni.2608.
2. Wong JM, de Souza R, Kendall CW, Emam A, Jenkins DJ. Colonic health: fermentation and short chain fatty acids. J Clin Gastroenterol 2006; 40:235-43; PMID:16633129; http://dx.doi.org/10.1097/00004836-200603000-00015.
3. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol 2004; 4:478-85; PMID:15173836; http://dx.doi.org/10.1038/nri1373.
4. Salminen S, Gibson GR, McCartney AL, Isolauri E. Influence of mode of delivery on gut microbiota composition in seven year old children. Gut 2004; 53:1388-9; PMID:15306608; http://dx.doi.org/10.1136/gut.2004.041640.
5. Dominguez-Bello MG, Costello EK, Contreras M, Magris M, Hidalgo G, Fierer N, Knight R. Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. Proc Natl Acad Sci U S A 2010; 107:11971-5; PMID:20566857; http://dx.doi.org/10.1073/pnas.1002601107.
6. Koenig JE, Spor A, Scalfone N, Fricker AD, Stombaugh J, Knight R, Angenent LT, Ley RE. Succession of microbial consortia in the developing infant gut microbiome. Proc Natl Acad Sci U S A 2011; 108 Suppl 1:4578-85; PMID:20668239; http://dx.doi.org/10.1073/pnas.1000081107.
7. Bezirtzoglou E, Tsiotsias A, Welling GW. Microbiota profile in feces of breast- and formula-fed newborns by using fluorescence in situ hybridization (FISH). Anaerobe 2011; 17:478-82; PMID:21497661; http://dx.doi.org/10.1016/j.anaerobe.2011.03.009.
8. Fallani M, Amarri S, Uusijarvi A, Adam R, Khanna S, Aguilera M, Gil A, Vieites JM, Norin E, Young D, et al. Determinants of the human infant intestinal microbiota after the introduction of first complementary foods in infant samples from five European centres. Microbiology 2011; 157:1385-92; PMID:21330436; http://dx.doi.org/10.1099/mic.0.042143-0.
9. Fallani M, Young D, Scott J, Norin E, Amarri S, Adam R, Aguilera M, Khanna S, Gil A, Edwards CA, et al. Intestinal microbiota of 6-week-old infants across Europe: geographic influence beyond delivery mode, breast-feeding, and antibiotics. J Pediatr Gastroenterol Nutr 2010; 51:77-84; PMID:20479681; http://dx.doi.org/10.1097/MPG.0b013e3181d1b11e.
10. Harmsen HJ, Wildeboer-Veloo AC, Raangs GC, Wagendorp AA, Klijn N, Bindels JG, Welling GW. Analysis of intestinal flora development in breast-fed and formula-fed infants by using molecular identification and detection methods. J Pediatr Gastroenterol Nutr 2000; 30:61-7; PMID:10630441; http://dx.doi.org/10.1097/00005176-200001000-00019.
11. Haarman M, Knol J. Quantitative real-time PCR assays to identify and quantify fecal Bifidobacterium species in infants receiving a prebiotic infant formula. Appl Environ Microbiol 2005; 71:2318-24; PMID:15870317; http://dx.doi.org/10.1128/AEM.71.5.2318-2324.2005.
12. Roger LC, Costabile A, Holland DT, Hoyles L, McCartney AL. Examination of faecal Bifidobacterium populations in breast- and formula-fed infants during the first 18 months of life. Microbiology 2010; 156:3329-41; PMID:20864478; http://dx.doi.org/10.1099/mic.0.043224-0.
13. Newburg DS. Oligosaccharides in human milk and bacterial colonization. J Pediatr Gastroenterol Nutr 2000; 30 Suppl 2:S8-17; PMID:10749396; http://dx.doi.org/10.1097/00005176-200003002-00003.
14. Garrido D, Dallas DC, Mills DA. Consumption of human milk glycoconjugates by infant-associated bifidobacteria: mechanisms and implications. Microbiology 2013; 159:649-64; PMID:23460033; http://dx.doi.org/10.1099/mic.0.064113-0.
15. Newburg DS. Neonatal protection by an innate immune system of human milk consisting of oligosaccharides and glycans. J Anim Sci 2009; 87:26-34; PMID:19028867; http://dx.doi.org/10.2527/jas.2008-1347.
16. Shoaf-Sweeney KD, Hutkins RW. Adherence, anti-adherence, and oligosaccharides preventing pathogens from sticking to the host. Adv Food Nutr Res 2009; 55:101-61; PMID:18772103; http://dx.doi.org/10.1016/S1043-4526(08)00402-6.
17. Kitaoka M. Bifidobacterial enzymes involved in the metabolism of human milk oligosaccharides. Adv Nutr 2012; 3:422S-9S; PMID:22585921; http://dx.doi.org/10.3945/an.111.001420.
18. Pavic AM, Hojsak I. Supplementation of prebiotics in infant formula. Nutr Dietary Supplements 2014; 6:69-74.
19. Mugambi MN, Musekiwa A, Lombard M, Young T, Blaauw R. Synbiotics, probiotics or prebiotics in infant formula for full term infants: a systematic review. Nutr J 2012; 11:81; PMID:23035863; http://dx.doi.org/10.1186/1475-2891-11-81.
20. Shoaf K, Mulvey GL, Armstrong GD, Hutkins RW. Prebiotic galactooligosaccharides reduce adherence of enteropathogenic Escherichia coli to tissue culture cells. Infect Immun 2006; 74:6920-8; PMID:16982832; http://dx.doi.org/10.1128/IAI.01030-06.
21. Sela DA, Li Y, Lerno L, Wu S, Marcobal AM, German JB, Chen X, Lebrilla CB, Mills DA. An infant-associated bacterial commensal utilizes breast milk sialyloligosaccharides. J Biol Chem 2011; 286:11909-18; PMID:21288901; http://dx.doi.org/10.1074/jbc.M110.193359.
22. Silfverdal SA, Bodin L, Olcen P. Protective effect of breastfeeding: an ecologic study of *Haemophilus influenzae* meningitis and breastfeeding in a Swedish population. Int J Epidemiol 1999; 28:152-6; PMID:10195681; http://dx.doi.org/10.1093/ije/28.1.152.
23. Simon PM, Goode PL, Mobasseri A, Zopf D. Inhibition of Helicobacter pylori binding to gastrointestinal epithelial cells by sialic acid-containing oligosaccharides. Infect Immun 1997; 65:750-7; PMID:9009338.
24. Wang B, Brand-Miller J. The role and potential of sialic acid in human nutrition. Eur J Clin Nutr 2003; 57:1351-69; PMID:14576748; http://dx.doi.org/10.1038/sj.ejcn.1601704.
25. Schauer R. Achievements and challenges of sialic acid research. Glycoconj J 2000; 17:485-99; PMID:11421344; http://dx.doi.org/10.1023/A:1011062223612.
26. Thum C, Cookson A, McNabb W, Roy CN, Otter D. Composition and enrichment of caprine milk oligosaccharides from New Zealand Saneen goat cheese whey. J Food Composition Analysis 2015; 42:30-7; http://dx.doi.org/10.1016/j.jfca.2015.01.022.
27. Van der Meulen R, Adriany T, Verbrugghe K, De Vuyst L. Kinetic analysis of bifidobacterial metabolism reveals a minor role for succinic acid in the regeneration of NAD^+ through its growth-associated production. Appl Environ Microbiol 2006; 72:5204-10; PMID:16885266; http://dx.doi.org/10.1128/AEM.00146-06.
28. Oliveira DL, Costabile A, Wilbey RA, Grandison AS, Duarte LC, Roseiro LB. In vitro evaluation of the fermentation properties and potential prebiotic activity of caprine cheese whey oligosaccharides in batch culture systems. Biofactors 2012; 6:440-9; http://dx.doi.org/10.1002/biof.1043.
29. Turroni F, Peano C, Pass DA, Foroni E, Severgnini M, Claesson MJ, Kerr C, Hourihane J, Murray D, Fuligni F, et al. Diversity of bifidobacteria within the infant gut microbiota. PLoS One 2012; 7:e36957; PMID:22606315; http://dx.doi.org/10.1371/journal.pone.0036957.
30. Kiyohara M, Tanigawa K, Chaiwangsri T, Katayama T, Ashida H, Yamamoto K. An exo-α-sialidase from bifidobacteria involved in the degradation of sialyloligosaccharides in human milk and intestinal glycoconjugates. Glycobiology 2011; 21:437-47; PMID:21036948; http://dx.doi.org/10.1093/glycob/cwq175.
31. Ward RE, Ninonuevo M, Mills DA, Lebrilla CB, German JB. In vitro fermentation of breast milk oligosaccharides by *Bifidobacterium infantis* and *Lactobacillus gasseri*. Appl Environ Microbiol 2006; 72:4497-9; PMID:16751577; http://dx.doi.org/10.1128/AEM.02515-05.
32. Cummings J, Pomare E, Branch W, Naylor C, Macfarlane G. Short chain fatty acids in human large intestine, portal, hepatic and venous blood. Gut 1987; 28:1221-7; PMID:3678950; http://dx.doi.org/10.1136/gut.28.10.1221.
33. Blaut M. Relationship of prebiotics and food to intestinal microflora. Eur J Nutr 2002; 41:i11-i6; PMID:12420111; http://dx.doi.org/10.1007/s00394-002-1102-7.
34. Cherbut C, Michel C, Lecannu G. The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats. J Nutr 2003; 133:21-7; PMID:12514261.
35. Dass N, John A, Bassil A, Crumbley C, Shehee W, Maurio F, Moore G, Taylor C, Sanger G. The relationship between the effects of short−chain fatty acids on intestinal motility in vitro and GPR43 receptor activation. Neurogastroenterol Motil 2007; 19:66-74;

PMID:17187590; http://dx.doi.org/10.1111/j.1365-2982.2006.00853.x.
36. Xiong Y, Miyamoto N, Shibata K, Valasek MA, Motoike T, Kedzierski RM, Yanagisawa M. Short-chain fatty acids stimulate leptin production in adipocytes through the G protein-coupled receptor GPR41. Proc Natl Acad Sci U S A 2004; 101:1045-50; PMID:14722361; http://dx.doi.org/10.1073/pnas.2637002100.
37. Bourriaud C, Robins R, Martin L, Kozlowski F, Tenailleau E, Cherbut C, Michel C. Lactate is mainly fermented to butyrate by human intestinal microfloras but inter-individual variation is evident. J Appl Microbiol 2005; 99:201-12; PMID:15960680; http://dx.doi.org/10.1111/j.1365-2672.2005.02605.x.
38. Gyorgy P, Norris RF, Rose CS. Bifidus factor. I. A variant of Lactobacillus bifidus requiring a special growth factor. Arch Biochem Biophys 1954; 48:193-201; PMID:13125589; http://dx.doi.org/10.1016/0003-9861(54)90323-9.
39. Xiao JZ, Takahashi S, Nishimoto M, Odamaki T, Yaeshima T, Iwatsuki K, Kitaoka M. Distribution of in vitro fermentation ability of lacto-N-biose I, a major building block of human milk oligosaccharides, in bifidobacterial strains. Appl Environ Microbiol 2010; 76:54-9; PMID:19854932; http://dx.doi.org/10.1128/AEM.01683-09.
40. Depeint F, Tzortzis G, Vulevic J, I'Anson K, Gibson GR. Prebiotic evaluation of a novel galactooligosaccharide mixture produced by the enzymatic activity of *Bifidobacterium bifidum* NCIMB 41171, in healthy humans: a randomized, double-blind, crossover, placebo-controlled intervention study. Am J Clin Nutr 2008; 87:785-91; PMID:18326619.
41. Goulas T, Goulas A, Tzortzis G, Gibson GR. Comparative analysis of four β-galactosidases from *Bifidobacterium bifidum* NCIMB41171: purification and biochemical characterisation. Appl Microbiol Biotechnol 2009; 82:1079-88; PMID:19099301; http://dx.doi.org/10.1007/s00253-008-1795-5.
42. Pokusaeva K, Fitzgerald GF, van Sinderen D. Carbohydrate metabolism in Bifidobacteria. Genes Nutr 2011; 6:285-306; PMID:21484167; http://dx.doi.org/10.1007/s12263-010-0206-6.
43. Asakuma S, Hatakeyama E, Urashima T, Yoshida E, Katayama T, Yamamoto K, Kumagai H, Ashida H, Hirose J, Kitaoka M. Physiology of consumption of human milk oligosaccharides by infant gut-associated bifidobacteria. J Biol Chem 2011; 286:34583-92; PMID:21832085; http://dx.doi.org/10.1074/jbc.M111.248138.
44. Barboza M, Sela DA, Pirim C, Locascio RG, Freeman SL, German JB, Mills DA, Lebrilla CB. Glycoprofiling bifidobacterial consumption of galacto-oligosaccharides by mass spectrometry reveals strain-specific, preferential consumption of glycans. Appl Environ Microbiol 2009; 75:7319-25; PMID:19801485; http://dx.doi.org/10.1128/AEM.00842-09.
45. Schell MA, Karmirantzou M, Snel B, Vilanova D, Berger B, Pessi G, Zwahlen MC, Desiere F, Bork P, Delley M, et al. The genome sequence of *Bifidobacterium longum* reflects its adaptation to the human gastrointestinal tract. Proc Natl Acad Sci U S A 2002; 99:14422-7; PMID:12381787; http://dx.doi.org/10.1073/pnas.212527599.
46. Sela DA, Chapman J, Adeuya A, Kim JH, Chen F, Whitehead TR, Lapidus A, Rokhsar DS, Lebrilla CB, German JB, et al. The genome sequence of *Bifidobacterium longum subsp. infantis* reveals adaptations for milk utilization within the infant microbiome. Proc Natl Acad Sci U S A 2008; 105:18964-9; PMID:19033196; http://dx.doi.org/10.1073/pnas.0809584105.
47. Ashida H, Miyake A, Kiyohara M, Wada J, Yoshida E, Kumagai H, Katayama T, Yamamoto K. Two distinct α-L-fucosidases from *Bifidobacterium bifidum* are essential for the utilization of fucosylated milk oligosaccharides and glycoconjugates. Glycobiology 2009; 19:1010-7; PMID:19520709; http://dx.doi.org/10.1093/glycob/cwp082.
48. Benthin S, Nielsen J, Villadsen J. Galactose Expulsion during Lactose Metabolism in *Lactococcus lactis subsp. cremoris* FD1 Due to Dephosphorylation of Intracellular Galactose 6-Phosphate. Appl Environ Microbiol 1994; 60:1254-9; PMID:16349233.
49. Bertino E, Peila C, Giuliani F, Martano C, Cresi F, Di Nicola P, Occhi L, Sabatino G, Fabris C. Metabolism and biological functions of human milk oligosaccharides. J Biol Regul Homeost Agents 2012; 26:35-8; PMID:23158512.
50. Sakurama H, Kiyohara M, Wada J, Honda Y, Yamaguchi M, Fukiya S, Yokota A, Ashida H, Kumagai H, Kitaoka M, et al. Lacto-N-biosidase Encoded by a Novel Gene of *Bifidobacterium longum Subspecies longum* Shows Unique Substrate Specificity and Requires a Designated Chaperone for Its Active Expression. J Biol Chem 2013; 288:25194-206; PMID:23843461; http://dx.doi.org/10.1074/jbc.M113.484733.
51. LoCascio RG, Desai P, Sela DA, Weimer B, Mills DA. Broad conservation of milk utilization genes in *Bifidobacterium longum subsp. infantis* as revealed by comparative genomic hybridization. Appl Environ Microbiol 2010; 76:7373-81; PMID:20802066; http://dx.doi.org/10.1128/AEM.00675-10.
52. Mei G-Y, Carey CM, Tosh S, Kostrzynska M. Utilization of different types of dietary fibres by potential probiotics. Can J Microbiol 2011; 57:857-65; PMID:21958046; http://dx.doi.org/10.1139/w11-077.
53. Falony G, Lazidou K, Verschaeren A, Weckx S, Maes D, De Vuyst L. In vitro kinetic analysis of fermentation of prebiotic inulin-type fructans by Bifidobacterium species reveals four different phenotypes. Appl Environ Microbiol 2009; 75:454-61; PMID:19011052; http://dx.doi.org/10.1128/AEM.01488-08.
54. Gibson GR, Wang X. Regulatory effects of bifidobacteria on the growth of other colonic bacteria. J Appl Bacteriol 1994; 77:412-20; PMID:7989269; http://dx.doi.org/10.1111/j.1365-2672.1994.tb03443.x.
55. Rossi M, Corradini C, Amaretti A, Nicolini M, Pompei A, Zanoni S, Matteuzzi D. Fermentation of fructooligosaccharides and inulin by bifidobacteria: a comparative study of pure and fecal cultures. Appl Environ Microbiol 2005; 71:6150-8; PMID:16204533; http://dx.doi.org/10.1128/AEM.71.10.6150-6158.2005.
56. Rada V, Petr J. A new selective medium for the isolation of glucose non-fermenting bifidobacteria from hen caeca. J Microbiol Methods 2000; 43:127-32; PMID:11121611; http://dx.doi.org/10.1016/S0167-7012(00)00205-0.
57. Kok RG, de Waal A, Schut F, Welling GW, Weenk G, Hellingwerf KJ. Specific detection and analysis of a probiotic Bifidobacterium strain in infant feces. Appl Environ Microbiol 1996; 62:3668-72; PMID:8837422.
58. Samarzija D, Sikora S, Redzepovic S, Antunac N, Havranek J. Application of RAPD analysis for identification of Lactococcus lactis subsp. cremoris strains isolated from artisanal cultures. Microbiol Res 2002; 157:13-7; PMID:11911609; http://dx.doi.org/10.1078/0944-5013-00126.
59. Perez G, Cardell E, Zarate V. Random amplified polymorphic DNA analysis for differentiation of Leuconostoc mesenteroides subspecies isolated from Tenerife cheese. Lett Appl Microbiol 2002; 34:82-5; PMID:11849499; http://dx.doi.org/10.1046/j.1472-765x.2002.01050.x.
60. Cocconcelli PS, Porro D, Galandini S, Senini L. Development of RAPD protocol for typing of strains of lactic acid bacteria and enterococci. Lett Appl Microbiol 1995; 21:376-9; PMID:8554764; http://dx.doi.org/10.1111/j.1472-765X.1995.tb01085.x.
61. Corroler D, Desmasures N, Gueguen M. Correlation between polymerase chain reaction analysis of the histidine biosynthesis operon, randomly amplified polymorphic DNA analysis and phenotypic characterization of dairy Lactococcus isolates. Appl Microbiol Biotechnol 1999; 51:91-9; PMID:10077825; http://dx.doi.org/10.1007/s002530051368.
62. Roy D, Sirois S. Molecular differentiation of Bifidobacterium species with amplified ribosomal DNA restriction analysis and alignment of short regions of the ldh gene. FEMS Microbiol Lett 2000; 191:17-24; PMID:11004394; http://dx.doi.org/10.1111/j.1574-6968.2000.tb09313.x.
63. Barrangou R, Altermann E, Hutkins R, Cano R, Klaenhammer TR. Functional and comparative genomic analyses of an operon involved in fructooligosaccharide utilization by *Lactobacillus acidophilus*. Proc Natl Acad Sci U S A 2003; 100:8957-62; PMID:12847288; http://dx.doi.org/10.1073/pnas.1332765100.
64. Turroni F, Bottacini F, Foroni E, Mulder I, Kim JH, Zomer A, Sanchez B, Bidossi A, Ferrarini A, Giubellini V, et al. Genome analysis of *Bifidobacterium bifidum* PRL2010 reveals metabolic pathways for host-derived glycan foraging. Proc Natl Acad Sci U S A 2010; 107:19514-9; PMID:20974960; http://dx.doi.org/10.1073/pnas.1011100107.
65. O'Brien M, Mitsuoka T. Quantitative fluorometric assay for rapid enzymatic characterization of *Bifidobacterium longum* and related bifidobacteria. Microbiol Immunol 1991; 35:1041-7; PMID:1808458; http://dx.doi.org/10.1111/j.1348-0421.1991.tb01626.x.
66. Hayashi H, Sakamoto M, Kitahara M, Y B. Molecular analysis of fecal microbiota in elderly individuals using 16S rDNA library and T-RFLP. Microbiol Immunol 2003; 47:557-70; PMID:14524616; http://dx.doi.org/10.1111/j.1348-0421.2003.tb03418.x.